The scientific challenges in moving from targeted to non-targeted mass spectrometric methods for food fraud analysis: A proposed validation workflow to bring about a harmonized approach

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Review

The scientific challenges in moving from targeted to non-targeted mass spectrometric methods for food fraud analysis: A proposed validation workflow to bring about a harmonized approach

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ABSTRACT

Background: Detecting and measuring food fraud is a challenging analytical task since a very wide range of food ingredients and types may be adulterated by numerous potential adulterants, many of which are yet unknown. To date most of the methods applied for the control of food fraud are targeted methods, which are focused on the detection of one or a few classes of known compounds.

Scope and approach: There is an increasing availability of solutions and applications based on high resolution mass spectrometry (HRMS), allowing parallel non-targeted approaches, novel compound identification and retrospective data analysis. For these types of methods sample-handling must be minimal to allow the inclusion of as many as possible chemical categories. However data-handling of such methods is much more demanding, together with the potential requirement to integrate multiplatform data as well as conducting data fusion. To allow the processing of massive amounts of information based on the separation techniques and mass spectrometry approaches employed, effective software tools capable of rapid data mining procedures must be employed and metabolomics based approaches does appear to be the correct way forward.

To verify the relevance of modelling results, appropriate model validation is essential for non-targeted approaches, confirming the significance of the chemical markers identified.

Key findings and conclusions: The present paper is devoted to review and assess the current state of the art with regards non-targeted mass spectrometry in food fraud detection within many food matrices and to propose a harmonized workflow for all such applications.

1. Introduction

Food fraud should be considered as a highly dynamic activity in which fraudsters aim to escape the regulatory and industry controls, for instance by hiding or changing the type of adulterants employed.

So far, most of the methods applied for the control of food fraud are targeted methods, which are focused on the detection of one or a few classes of compounds. In many cases the extraction procedures are complex and expensive, but enable to lower the analytes detection limits (up to sub ppt-levels) also in complex matrices (Kaufmann, Butcher, Maden, Walker, & Widmer, 2015). However, recent advances in mass spectrometry, mainly high resolution mass spectrometry (HRMS) together with improvements in user friendly software, have allowed non-targeted approaches to be developed (Kaufmann, 2014). In this type of methods, target inclusion lists are not used, since the molecules to be detected are not known *a priori*.

Indeed, the analysis aims to study the global sample fingerprint itself.

The increasing popularity of HRMS is mainly due to the introduction of benchtop instruments, such as Time-of-Flight (ToF) and Orbitrap, and the advantages of using full-scan acquisition mode with high sensitivity, combined with high resolving power (up to 100,000 FWHM) and accurate mass measurement (< 5 ppm). In addition, the acquisition of high resolution full scanned data permits the combination of target analysis with screening of non-target compounds, novel compound identification, and retrospective data analysis. Moreover, a broad range of m/z values can be recorded simultaneously, without any target compounds list and individual optimization (Kaufmann, 2012). In most cases a generic sample preparation is performed which allows, in principle, a very broad view of any potential compounds of interest.

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Therefore, the advantages of applying analytical methods not focused on a narrow group of targeted analytes are very evident, underling the need to widen the range of monitored compounds to combat the complexities of food adulteration.

Besides the previously mentioned advantages of using non-targeted approaches, some critical aspects have also to be taken into account. Non-targeted data-handling is much more demanding compared to that required in classical targeted approaches. In targeted analysis, results are usually evaluated compound-by-compound using univariate statistics. By contrast, the data collected for non-targeted approaches typically needs to be evaluated using multivariate statistical models (Riedl, Esslinger, & Fauhl-Hassek, 2015). In addition, the huge diversity in data processing workflows applied through the available scientific literature makes the evaluation of method performance extremely challenging. An agreed, harmonized and 'official' workflow for development and validation of non-targeted methods is very much required. A science-based approach was presented by Alewijn and co-workers (Alewijn, van der Voet, & van Ruth, 2016) in which a validation roadmap is described starting from the criteria for the selection of suitable samples for the training set, passing through the identification of the most appropriate analytical methods and continuing with a description of some initial validation steps (i.e. repeatability, permutation tests). Subsequently, a cross-validation of the training set and the prediction of an external set of samples are suggested as crucial points for a robust validation.

This publication was followed by a preliminary attempt of untargeted analysis harmonization, which has been recently suggested by the US Pharmacopoeia (USP Pharmacopeial Convention., 2016, pp. 2053–2067). In this document, great attention is placed on the criteria that must be used to build-up a "reference" and a "test" samples set able to provide a reliable predictive model. The concepts of sensitivity and specifity rate are introduced, together with the idea that the evaluation of the receiver operating characteristic (ROC) curves could represent an important tool to assess the goodness of the method. According to this guideline, after the selection of the analytical approach and of the appropriate modelling technique, the method should be developed and optimized with the "reference set" and the "test set". Subsequently, sensitivity and specificity rates should be evaluated together with the ROC curves. If the criteria are fulfill, the method should be validated with new samples and, after its release for use, a monitoring process should be put in place with the aim to check the reliability of the method over a period of time (USP Pharmacopeial Convention., 2016, pp. 2053–2067).

This guideline is very welcome but the process should be considered to only be at the beginning. Furthermore, USP guideline is generic and so is not able to provide suggestions specific for each analytical technique.

Therefore, this article has been aimed to assess the current state of the art on non-targeted mass spectrometry in food fraud detection and to propose a harmonized workflow.

1.1. Literature overview

Few keywords ("non-targeted", "mass spectrometry", "authenticity", "fraud" and the target matrix) were mainly set and used to obtain a representative set of studies from the Scopus database. A wide range of food matrices were considered, while mass spectrometry was the only analytical technique considered. Searches included articles published from 2011 to 2017. Altogether, 49 articles were evaluated, from 16 different scientific journals.

As detailed in Tables 1 and 2, information available in literature on food fraud deals with a large number of commodities, including meat, spices, wine and cereals. The most commonly addressed issues that pertain to geographical origin protection, proof of the authenticity, followed by the detection of different types of adulterations (i.e mixtures, dilutions, substitutions).

As detailed in Table 3 (that resumes the validation parameters verified by the authors of each of the articles selected for this review) non-targeted mass spectrometry workflows appear to be non-standardized to date. Experimental design as well as crucial parameters (i.e. number of samples, number of replicates, sample sources) quite often are not clearly detailed in the articles or completely absent in some cases.

Another challenging issue that should be addressed in the future is the representativeness of the samples used to build the models. Authentic samples must be provided by certified producers and/or guaranteed by official center (i.e. PDO). In fact, the use of non-authentic samples could well result to a misleading classification model.

From an analytical point of view, sample preparation is usually simple and rapid: every cleanup step could potentially decrease the number of detected compounds, with a depletion of the chromatographic fingerprint (Vuckovic, 2012). Liquid chromatography coupled to mass spectrometry (especially high resolution mass spectrometry) is the most widely used approach, followed by Direct Analysis in Real Time (DART)-MS, GC-MS, Proton Transfer Reaction (PTR)-MS and other techniques.

Generally, accepted chemometric models are used for data processing, with the exception of proteomic studies. Prediction clusters (multivariate models aiming to predict class membership with no marker selection) and discriminative model with markers identification (that ends with the identification of significant compounds responsible for class membership) are the chemometric approaches mainly presented in the literature so far.

The most applied unsupervised technique is the Principal Component Analysis (PCA), while Orthogonal Partial Least Square – Discriminant Analysis ((O)PLS-DA) and Linear Discriminant Analysis (LDA) are the most commonly employed supervised models. The identification of markers represents the most challenging and time-consuming step. Indeed, quite often they remain unknown (only the *m/z* values are provided) or only a tentative identification is presented (Kind & Fiehn, 2007). Only a few studies followed the criteria proposed by the Standard Initiative in metabolomics (Sumner et al., 2007) as subsequently amended and supplemented (Schymanski et al., 2014), performing a first level identification, thus by unambiguously confirming the identity with Reference Standards injection.

Most of the investigated papers reached a "Level II" that corresponds to compound identified by HRMS/MS spectra matching with literature or libraries ("Level IIa") or by diagnostic evidence when only one structure fits the experimental data but no standard or literature information is available for confirmation ("Level IIb").

On the other hands, metabolites are considered putatively characterized when evidence exists for possible structures but there is not enough information for one exact structure only ("Level III").

Finally, unknown compounds can be classified as "Level IV" when an unequivocal molecular formula can be assigned but a structure cannot be hypothesized and as "Level V" when a specific exact mass is important for the study but no information are useful to identify the compound (Sumner et al., 2007) (Schymanski et al., 2014). Another critical step is represented by the validation of chemometric models, essential to assess their reliability, but quite often this step is not undertaken in published studies. When applied, different and sometimes incomplete validation approaches are presented, suggesting, once again, the urgent need for a harmonization approach to method validation. These considerations will be further detailed in the following paragraphs.

1.2. Design of experiments (DOEs)

One important point is to define the study question being asked (i.e. geographical origin, organic or conventional regimen, percentage of adulteration). After the definition of a question, the study design has to be planned, involving the choice of which samples to collect and a reliable procedure for handling and measuring the samples minimizing

Table 1
Commodities, number of papers, instrumental method and chemometric method used in the articles that meet the selection criteria used for the literature search.

Commodity	Sub-commodity	Number of papers	Method										Chemometric Method								
			LC-MS	LC-HRMS	HRMS	DART-MS	REIMS-HRMS	MALDI-HRMS	MALDI-LRMS	LESA-HRMS	SPME-MS	PTR-MS	GC-MS	PCA	(O) PLS-DA	SIMCA	(L)DA	k-NN	(H)CA	k-Means	PCO	CP-ANN
EVOO		4	2					1				1		2	3			1	1			1
FISH		1	1																			
Balsamic Vinegar		1							1					1								
Coffee		2			1							1		2	2							
Cheese		2		1								1		1	1	1						
Tomatoes		2				1							1	2			1					
Fruit Juices		3	1	3										3	2	1	1					
Honey		5		3								1	1	5	3		1	1				
Carrot		1		1										1	1							
Meat		8	2	2		2	1			1		1		4	1	1	2					
Herbs and spices	Saffron	3		2		1							1	3	2					1		1
	Oregano	1		1										1	1							
Wine		4		2		1					1		1	4	3		1					
Spirit drinks	Whisky	3		1	2									2	1		1					
Sea Buckthorn Oil		1		1		1																
Soybean		1		1		1								1	1							
Cereals	Wheat	6	1	4									1	4	3	1			1		1	
Tiger nut		1												1	1							

PCA: Principal component analysis, (O)PLS-DA: (orthognol) partial least squares discriminant analysis, (L)DA: (Linear) discriminant analysis, SIMCA: Soft independent modelling by class analogy, k-NN: k-Nearest-Neighbors, (H)CA: (hierarchical) cluster analysis, PCO: principal coordinates analysis, CP-ANN: Counter propagation artificial neural network, LC-MS: liquid chromatography-mass spectrometry, LC-HRMS. liquid chromatography-high resolution mass spectrometry, HRMS: high resolution mass spectrometry, DART-MS. direct analysis in real time-mass spectrometry, REIMS-HRMS: rapid evaporative ionization mass spectrometry, MALDI-HRMS: matrix-assisted laser desorption/ionization-high resolution mass spectrometry, MALDI-LRMS: matrix-assisted laser desorption/ionization-low resolution mass spectrometry, LESA-HRMS: liquid extraction surface analysis-high resolution mass spectrometry, SPME-MS. Solid phase micro extraction-mass spectrometry, PTR-MS: proton transfer reaction–mass spectrometry, GC-MS: gas chromatography-mass spectrometry.

Table 2
Summary of analytical approaches of the articles that meet the selection criteria, related to different raw materials.

Reference	Objective	Commodity	Fraud investigated	Sample prep	Method	Data pre treatment	Software	Chemometric methods	Classifications	Output	Sample source	Total No Samples
(Kalogiouri, Alygizakis, Aalizadeh, & Thomaidis, 2016)	Authenticity	Extra virgin olive oil	Defective olive oil	Liquid-liquid extraction, centrifugation and filtration	LC-HRMS	N.A.	XCMS	PLS-DA; CP-ANN	2 - classes	Predictive clusters	From the Kolovi and Adramitiani varieties	22
(Di Girolamo et al., 2015)	Adulteration	Extra virgin olive oil	Corn oil addition	Liquid-liquid extraction and centrifugation	MALDI-HRMS	Normalization	Biotyper 3.1, ClinProTools	HCA; PCA	2 - classes	Predictive clusters	Pure oil samples and home maid mixtures	8
(Gil-Solsona et al., 2016)	Geographical origin discrimination	Extra virgin olive oil	False geographical origin declaration	Liquid-liquid extraction (polar fraction)/dilution (non polar fraction)	LC-HRMS	Mean centering normalization, log2 transformation	EZInfo	PCA; PLS-DA; OPLS-DA	2-classes	Disciminative	From local cooperatives	105
(Ruiz-Samblás et al., 2012)	Monovarietal extra virgin olive oil identification	Extra virgin olive oil	False monovarietal extra virgin olive oil declaration	No sample prep	PTR-MS	Autoscaling	MATLAB	PLS-DA	5-classes	Predictive clusters	Samples purchased from common markets in Spain	30
(Wulff, Nielsen, Deelder, Jessen, & Palmblad, 2013)	Authenticity	Fish	N.A.	Proteins extraction and digestion	LC-MS	N.A.	DataAnalysis	N.A.	N.A.	N.A.	Samples from different fishes species	69
(Guerreiro, de Oliveira, Ferreira, & Catharino, 2014)	Adulteration	Balsamic Vinegar of Modena	Addition of other vinegars	Dilution	MALDI-LRMS	N.A.	Unscrambler	PCA	6-classes	Disciminative	All vinegars purchased from commercial establishments	N.A.
(Garrett et al., 2013)	Arabica Coffee Geographical origin discrimination	Coffee	False geographical origin declaration	Solid-Liquid extraction and centrifugation	HRMS	Pareto scaling	MetaboAnalyst	PCA; PLS-DA	2-classes	Disciminative	Coffe samples from a field study	8 for each region
(Özdestan et al., 2013)	Specialty coffees discrimination	Coffee	False organic declaration	No sample prep	PTR-MS	Autoscaling	Pirouette	PCA; PLS-DA	2-classes	Predictive clusters	Samples collected from retail outlests in the Netherlands	110
(Popping, De Dominicis, Dante, & Nocetti, 2017)	Geographical origin of Paramigiano Reggiano	Cheese	False geographical origin declaration	Solid-Liquid extraction	LC-HRMS	Pareto scaling	SIMCA	SIMCA	2-classes	Predictive clusters	Samples commercially available	84
(Galle et al., 2011)	Geographical origin of PDO cheese of Leiden	Cheese	False geographical origin declaration	No sample prep	PTR-MS	Autoscaling	Pirouette; STATISTICA	PCA; PLS-DA	2-classes	Predictive clusters	Samples purchased from supermarkets and from registered cheese farms	59
(Figueira, Câmara, Pereira, & Câmara, 2014)	Cultivars discrimination	Tomatoes	N.A.	Dilution and SPME extraction	GC-MS	Normalization	SPSS	PCA	5-classes	Predictive clusters	Samples obtained from local producers	5
(Novotná et al., 2012)	Organic and conventional farming discrimination	Tomatoes	False organic declaration	Solid-Liquid extraction and centrifugation	DART-MS	Normalization	statistiXL	PCA; LDA	2-classes	Predictive clusters	Samples obtained from a field study	40

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Table 2 (*continued*)

Reference	Objective	Commodity	Fraud investigated	Sample prep	Method	Data pre treatment	Software	Chemometric methods	Classifications	Output	Sample source	Total No Samples
(Jandric et al., 2014)	Grapefruit and Oranje juices authenticity	Fruit Juices	Low price fruit juices addition	Centrifugation and filtration	LC-HRMS	N.A.	EZinfo	PCA; OPLS-DA	6-classes for each juice	Disciminative	Fruits purchased from Austrian markets	28
(Jandric et al., 2017) 2017	Indian citrus fruit/ fruit juices adulteration	Fruit Juices	N.A.	Centrifugation and filtration	LC-HRMS	Pareto Scaling	SIMCA	PCA; SIMCA; OPLS-DA	4-classes	Disciminative	Fruit juices produced in the laboratory from authentic citrus juices	N.A.
(Vaclavik, Schreiber, Lacina, Cajka, & Hajslova, 2012)	Authenticity	Fruit Juices	Addition of low price juices to expensive juices	Centrifugation. filtration and dilution	LC-MS/ LC-HRMS	Pareto scaling	MarkerView, statistiXL	PCA; LDA	3-classes	Disciminative	Purchased from Czech and Canadian markets	84
(Li et al., 2017)	Single/multi floral and geographical origin authenticity	Honey	N.A.	Liquid-liquid extraction, Centrifugation and filtration	LC-HRMS	Range Scaling	SIMCA	PCA; PLS-DA	2-classes	Disciminative	Honey samples collected from apiaries	210
(Silva et al., 2017)	Sugarcane honey authenticity	Honey	N.A.	No sample prep, direct extraction with SPME	GC-MS	N.A.	SPSS; STATISTICA	PCA; LDA	2-classes	Discriminative	Samples were from Madeira Island, Portugal	16
(Jandrić et al., 2015)	Floral origin authentication	Honey	N.A.	Dilution, centrifugation and filtration	LC-HRMS	Pareto scaling	SIMCA	PCA; OPLS-DA	4-classes	Disciminative	Samples obtained from honey producers	83
(Spiteri et al., 2016)	Botanical origin	Honey	N.A.	Liquid-liquid extraction (Orbitrap); QuEChERS (TOF-MS)	LC-HRMS	Log transformation and Pareto Scaling	R; Matlab	PCA; PLS-DA	5-classes	Discriminative	Samples collected from different supermarkets	56
(Kus & van Ruth, 2015)	Floral origin authentication	Honey	N.A.	No sample prep	PTR-MS	Autoscaling	Pirouette	PCA; k-NN	6-classes	Predictive clusters	Samples collected in Poland	62
(Cubero-Leon, De Rudder, & Maquet, 2018)	Organic authentication	Carrot	Conventional carrots in declared organic carrots	Solid-Liquid extraction, centrifugation and filtration	LC-HRMS	Pareto scaling	SIMCA	PCA; OPLS-DA	2-classes	Predictive clusters	Samples from field study	210
(Balog et al., 2016)	Identification of the species of origin for meat products	Meat	Addition of not declared meat species	No sample prep, direct analysis with REIMS	REIMS-HRMS	N.A.	MATLAB	PCA; LDA	Different classifications are explored	Predictive clusters	Samples from regional abattoir	15 bovine and 5 equine samples. All samples were divided into four pieces, and a total of 30 sampling points were taken in four separate experiments. Total: 600 sampling points
(Vaclavik et al., 2011)	Animal fats (lard and beef tallow) authentication	Meat	Pork lard and beef tallow admixtures	Solid-Liquid extraction and centrifugation	DART-HRMS	Constant row sum transformation	StatistiXL, Statistica	PCA; LDA	2-classes	Predictive clusters	Samples purchased from the retail market	29
		Meat	Not declared meat mixtures	Solid-Liquid extraction,	LC-MS	N.A.	N.A.	N.A.	N.A.	N.A.	Samples purchased from	23

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Table 2 (*continued*)

Reference	Objective	Commodity	Fraud investigated	Sample prep	Method	Data pre treatment	Software	Chemometric methods	Classifications	Output	Sample source	Total No Samples
(von Bargen, Brockmeyer, & Humpf, 2014)	Horse and pork detection in highly processed food			digestion, desalting							the market and in-house processed samples	
(Prandi et al., 2017)	Highly processed meat authentication	Meat	Fraudulent beef and pork meat mixtures in Bolognese sauce	Proteins extraction and digestion	LC-MS	N.A.	N.A.	N.A.	N.A.	N.A.	Samples prepared in a pilot plant	N.A.
(Cajka, Danhelova, Zachariasova, Riddellova, & Hajslova, 2013)	Chicken feeding discrimination	Meat	Bone meal addition to chicken feed	Solid-Liquid extraction and centrifugation	DART-MS	Constant row sum transformation; Pareto scaling	SIMCA	PCA; OPLS-DA	2-classes	Predictive clusters	Chickens grown under controlled conditions	116 (chicken) 24 (feed)
(Sarah et al., 2016)	Discrimination of pork from beef, chevon from chicken in thermally processed meat	Meat	Fraudulent meats mixtures	Proteins extraction and digestion	LC-HRMS	N.A.	N.A.	N.A.	N.A.	N.A.	Authentic meat purchased directly from commercial abattoirs	4
(Montowska, Alexander, Tucker, & Barrett, 2015)	Beef, pork, horse, chicken and turkey meat authentication in processed samples	Meat	Fraudulent meats mixtures	Protein digestion and centrifugation	LESA-HRMS	N.A.	N.A.	N.A.	N.A.	N.A.	Meat products purchased at supermarket	18
(Oliveira, Alewijn, Boerrigter-Eenling, & van Ruth, 2015)	Discrimination of Conventional, Free Range, and Organic pork meat	Meat	Fraudulent organic declaration	No sample prep (PTR-MS); fat melted and dissolved in DCM (LC-HRMS)	LC-HRMS, PTR-MS	Autosaling (LC-HRMS); Pareto scaling (PTR-MS)	Pirouette	PCA, SIMCA	3-classes	Predictive clusters	Samples purchased by a meat producing company in the Netherlands	41
(Rubert, Lacina, Zachariasova, & Hajslova, 2016)	Authentic PDO saffron vs unknown samples	Saffron	False origin declaration	Solid-Liquid extraction and centrifugation	DART-MS; LC-HRMS	Pareto scaling	MarkerView, SIMCA	PCA; OPLS-DA	2-classes	Discriminative	Samples purchased from markets.	44
(Aliakbarzadeh, Sereshti, & Parastar, 2016)	Geographical regions origin of Iranian saffron authentication	Saffron	False origin declaration	Ultrasound-assisted solvent extraction (UASE) and dispersive liquid–liquid microextraction (DLLME)	GC-MS	MCR–ALS technique for chromatographic peaks resolving	MATLAB	PCA; k-Means; CP-ANN	5-classes	Discriminative	Sample obtained from producers and agricultural research centers	17
(Guijarro-Díez, Nozal, Marina, & Crego, 2015)	Authenticity certification for Spanish and Iranian Saffron	Saffron	Fraudulent mixtures	Solid-Liquid extraction, centrifugation, dilution and filtration	LC-HRMS	Log transformation and Pareto Scaling	SIMCA	PCA; PLS-DA; OPLS-DA	2-classes	Discriminative	Samples provided by "Carmencita" company	20
(Black, Haughey, et al., 2016)	Adulteration	Oregano	Different adulterants (i.e.addition of olive or myrtle leaves etc)	Solid-Liquid extraction, centrifugation and filtration	LC-HRMS	Mean centering and Pareto scaling	SIMCA	PCA; OPLS-DA	6-classes	Discriminative	Samples purchased from markets.	N.A.
(Vaclavik, Lacina, Hajslova, &	Discrimination of red wine varieties	Wine	N.A.	No sample prep, direct injection in HPLC	LC-HRMS	Mean centering and log transformation	Mass Profiler Professional	PCA; PLS-DA	3-classes	Discriminative	Samples purchased from retail markets.	45

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Table 2 (*continued*)

Reference	Objective	Commodity	Fraud investigated	Sample prep	Method	Data pre treatment	Software	Chemometric methods	Classifications	Output	Sample source	Total No Samples
Zweigenbaum, 2011)												
(Springer et al., 2014)	Wine botanical origin verification	Wine	False botanical origin declaration	No sample prep, direct extraction with SPME	GC-MS	Square root transformation, row and column scaling	SIMCA	PCA; PLS-DA; OPLS-DA	4-classes	Predictive clusters	Commercial wine samples	198
(Rubert et al., 2014)	Wine varietal authentication	Wine	N.A.	Direct injection for fingerpirnt; Liquid-liquid extraction for Polyphenol profiling	LC-HRMS; DART-MS	Total area sum normalization	MarkerView, SIMCA	PCA; OPLS-DA	3-classes (white varieties), 3-class (red varieties)	Discriminative	Commercial wines	343
(Ziółkowska, ìWąsowicz, & Jeleń, 2016)	Grape variety and geographical origin discrimination	Wine	False grape variety and false geographical origin declaration	vial SPME extraction	SPME-MS	Autoscaling and log transformation	Statistica	PCA; LDA	Varieties: 3-classes (white),2-classes (red). Origin: 9-classes (white) 5-classes (red)	Predictive clusters	Wines purchased in wine shops	79
(Kew, Goodall, Clarke, & Uhrín, 2017)	Characterization of Whiskie types	Spirit Drinks-whisky	Whiskie adulteration	Dilution	HRMS	Mean centering and scaling to unit variance	SIMCA	PCA; OPLS-DA	2-classes	Discriminative	Commercial samples	85
(Collins, Zweigenbaum, & Ebeler, 2014)	Characterization of Whiskies type and aging	Spirit Drinks-whisky	Whiskey producers and ages	Direct injection	LC-HRMS	N.A.	XLSTAT	LDA	N.A.	Discriminative	Commercial samples	63
(Garcia et al., 2013)	Whiskies adulteration detection	Spirit Drinks-whisky	Addition of not declared components	Dilution	HRMS	N.A.	Pirouette	PCA	5-class (different brands); 2-class (adulterated and not adulterated)	Predictive clusters	Commercial samples	80
(Hurkova, Rubert, Stranska-Zachariasova, & Hajslova, 2017)	Adulteration	Sea Buckthorn Oil	Addition of sunflower oil	Liquid-Liquid extraction, centrifugation.	DART-MS; LC-HRMS	N.A.	N.A.	N.A.	N.A.	Discriminative	Samples purchased at supermarket	N.A.
(Hrbek et al., 2017)	GMO and Non-GMO Soybean discrimination	Soybean	Presence of GMO soybean	Solid-Liquid extraction, centrifugation and filtration	DART-MS; LC-HRMS	Pareto scaling	SIMCA	PCA; OPLS-DA	2-classes	Discriminative	Samples from field study	49
(Pastor et al., 2016)	Small grains and corn flour authentication	Cereals - Wheat	Fraudulent mixed flour bakery products	Solid-Liquid extraction, centrifugation	GC-MS	N.A.	PAST	PCO; CA	2-classes	Discriminative	Samples from field study	40
(Prandi et al., 2012)	Durum wheat authenticity	Cereals - Wheat	Addition of not declared common wheat	Enzimatic cleavage	LC-MS	N.A.	N.A.	N.A.	N.A.	Discriminative	Analysis of wheat varieties and commercial samples	N.A.
(Geng, Harnly, & Chen, 2016)	Whole grain and refined wheat flours discrimination in bread	Cereals-Wheat	Refined wheat addition to whole wheat for bread preparation	Solid-liquid extraction, centrifugation and filtration	LC-HRMS	N.A.	SIMCA	PCA; SIMCA	2-classes	Discriminative	Samples purchased from local grocery stores or online	72
(Righetti et al., 2016)	Ancient Triticum species characterization	Cereals-Wheat	N.A.	Solid-liquid extraction and centrifugation	LC-HRMS	Total area sum normalization, Pareto scaling	MarkerView, SIMCA	PCA; PLS-DA; OPLS-DA	3-classes	Discriminative	Wheat from field study	77

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Table 2 (*continued*)

Reference	Objective	Commodity	Fraud investigated	Sample prep	Method	Data pre treatment	Software	Chemometric methods	Classifications	Output	Sample source	Total No Samples
(Matthews et al., 2012)	Common and durum wheat characterization	Cereals-Wheat	N.A.	Solid-liquid extraction and centrifugation	LC-HRMS	Pareto scaling	SIMCA	PCA; OPLS-DA; HCA	3-classes	Discriminative	Commercial samples	45
(Righetti et al., 2018)	Common and durum wheat discrimination	Cereals-Wheat	Fraudulent common wheat addition	Solid-liquid extraction and centrifugation	LC-HRMS	Pareto scaling	SIMCA, SPSS	PCA; OPLS-DA	2-classes	Discriminative	Wheat samples from a field study	225
(Rubert, Hurkova, Stranska, & Hajslova, 2017)	Discrimination between PDO Valencian tiger nut and African tiger nut	Tiger nut	False PDO Valencian tiger nut declaration	Solid-Liquid extraction, centrifugation	LC-HRMS	Pareto scaling	MarkerView, SIMCA	PCA; OPLS-DA	2-classes	Discriminative	Xufa de València	45

PCA: Principal component analysis, (O)PLS-DA: (orthogonal) partial least squares discriminant analysis (L)DA: (Linear) discriminant analysis, SIMCA: Soft independent modelling by class analogy, k-NN: k-Nearest-Neighbors, (H)CA: (hierarchical) cluster analysis, PCO: principal coordinates analysis, CP-ANN: Counter propagation artificial neural network LC-MS: liquid chromatography-mass spectrometry, LC-HRMS. liquid chromatography-high resolution mass spectrometry, HRMS: high resolution mass spectrometry, DART-MS. direct analysis in real time-mass spectrometry, REIMS-HRMS: rapid evaporative ionization mass spectrometry, MALDI-HRMS: matrix-assisted laser desorption/ionization-high resolution mass spectrometry, MALDI-LRMS: matrix-assisted laser desorption/ionization-low resolution mass spectrometry, LESA-HRMS: liquid extraction surface analysis-high resolution mass spectrometry, SPME-MS. Solid phase micro extraction-mass spectrometry, PTR-MS: proton transfer reaction–mass spectrometry, GC-MS: gas chromatography-mass spectrometry, N.A.: information not available in the paper.

Table 3
Summary of the validation parameters verified in each of the articles selected for this review.

Reference	Extraction replicates	QC	Performance	Marker identification	Validation	ROC curve	Representative (provenance/ verification of state)	Reference samples	Test samples	Validation set	Confirmed by ref/ secondary method	Mixed adulterants
(Kalogiouri et al., 2016)	N.A.	yes	N.A.	yes	Model validation with 4 samples used to evaluate the external accuracy. Markers confirmed with the injection of reference standards	yes	Samples produced in the 2014–2015 period and cultivated in different regions on Lesvos	Standard defective olive oils from the International Olive Council, other samples from the Kolovi and Adramitiani varieties	From the Kolovi and Adramitiani varieties	15	N.A.	N.A.
(Di Girolamo et al., 2015)	4 replicates for all the samples	N.A.	N.A.	N.A.	Mixtures analyses. Two of them treated as blind samples and analyzed by an other investigator	N.A.	4 different italian cultivars; different commercial brands	Pure oil samples	In-house prepared mixtures	N.A.	N.A.	0.5%, 1%, 5%, 10%, 20% w/w addition of Corn oil to EVOO
(Gil-Solsona et al., 2016)	N.A.	yes	N.A.	yes	Cross- validation; analysis of 15 external samples	N.A.	Samples from 6 different spanish regions	Samples certified by the Spanish Agriculture ministry	Samples collected in different seasons	15	N.A.	N.A.
(Ruiz-Samblás et al., 2012)	Samples analyzed in triplicate	N.A.	N.A.	yes	30% of samples used for internal cross validation and external validation	N.A.	Five different varieties of olive fruit (Arbequina, Cornicabra, Frantoio, Hojiblanca, and Picual) cultivated in Spain and Italy	N.A.	N.A.	N.A.	N.A.	N.A.
(Wulff et al., 2013)	4 technical replicates	N.A.	N.A.	N.A.	Other samples prepared at different times, using different LC systems with different columns	N.A.	All included species were assigned by experienced zoologists	N.A.	N.A.	47	N.A.	N.A.
(Guerreiro et al., 2014)	N.A.	N.A.	N.A.	yes	N.A.	N.A.	Other vinegars: red wine, white wine, apple and alcohol	PGI certification from the region of Modena (Italy)	N.A.	N.A.	N.A.	10%, 20% and 50% addition of each vinegar to the balsamic vinegar
(Garrett et al., 2013)	All the samples in triplicate	N.A.	PCA: 2PCs 66% variance	yes	Leave one-out cross-validation	N.A.	Arabica coffee cultivars grown in two brasilian regions under the same edaphoclimatic conditions	N.A.	/	/	N.A.	N.A.
(Özdestan et al., 2013)	All the samples in triplicate	N.A.	PCA: 2PCs 88.5% variance	yes	10-fold cross-validation	N.A.	Samples obtained in the last two months of 2011/early 2012. Organic and conventional samples originated from Southand Central America, Africa and Asia. A group of samples had a mixture of origins.	All groups of samples included fair trade certified coffees (54 samples in total)	N.A.	N.A.	N.A.	N.A.

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Table 3 (*continued*)

Reference	Extraction replicates	QC	Performance	Marker identification	Validation	ROC curve	Representative (provenance/ verification of state)	Reference samples	Test samples	Validation set	Confirmed by ref/ secondary method	Mixed adulterants
(Popping et al., 2017)	All the samples in triplicate	yes	PCA-Class correctly classified 87.5% of samples	no	Leave one-out cross-validation; classification of an external set of samples	N.A.	Different geographical origin and ageing	A group of Parmigiano Reggiano samples were certified as PDO	Blind samples	32	N.A.	N.A.
(Galle et al., 2011)	Analysis in triplicate	N.A.	PCA: 2PCs 45.6% variance	yes	Leave one-out cross-validation	N.A.	Artisanal farmers' cheese PDO samples and commercial Dutch cumin cheese samples of varying brands without PDO protection	All the artisanal farmers' cheese of Leiden samples were certified as PDO	N.A.	N.A.	N.A.	N.A.
(Figueira et al., 2014)	All the samples in triplicate	N.A.	PCA: 2PCs 87.4% variance	yes	Reference standards injection for a group of marker compounds; Leave one-out cross-validation	N.A.	All cultivars grew at southeast of Madeira Island (Portugal) and cultivated under similar conditions	N.A.	N.A.	N.A.	N.A.	N.A.
(Novotná et al., 2012)	N.A.	N.A.	N.A.	yes	Leave one-out cross-validation	N.A.	Four locations, two farming methods, five varieties, two harvest years	N.A.	N.A.	N.A.	N.A.	N.A.
(Jandric et al., 2014)	N.A.	yes	Different results according to the mixtures analyzed	yes	Some markers confirmed with reference standards. Markers detected in the admixtures samples	N.A.	N.A.	N.A.	In-house prepared mixtures	N.A.	N.A.	Pineapple juice adulterated with orange, apple, grapefruit and clementine were prepared at 1%, 5%, 10% and 15% adulteration. Orange juice was adulterated with apple, grapefruit and clementine at the same adulteration levels.
(Jandric et al., 2017)	3 replicates	N.A.	PCA: 2PCs 80,1% variance	yes	Leave one-out cross-validation; some markers confirmed with the injection of the reference standards. Subsequent Target method to detect markers	N.A.	Different citrus varieties grown in separate rows	Authentic citrus fruits obtained from the Indian Agriculture Research Institute.	Mixtures of citrus fruits juice adulterated with other juices	N.A.	N.A.	Mixtures of citrus fruits juice adulterated with each other were prepared at 1, 2, 5 and 10%
(Vaclavik et al., 2012)	Mixtures in triplicate; single preparation for the pure samples	N.A.	PCA: 2PCs 45% variance (pos) 30,6% variance (neg)	yes	Leave one-out cross-validation and Sevenfold cross-validation LDA model created also with the mixtures	N.A.	Collected samples represented both fresh-pressed juices and juices prepared from concentrate and were produced in various countries	N.A.	In-house prepared mixtures	N.A.	N.A.	10%, 15%, 25%, 50%, 75% of apple juice addition to oragnge juice. 10%, 15%, 25%, 50%, 75%, 90% of grapefruit addition to orange juice
(Li et al., 2017)	N.A.	yes	N.A.	yes	N.A.	N.A.		N.A.	N.A.	N.A.	N.A.	N.A.

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Table 3 (*continued*)

Reference	Extraction replicates	QC	Performance	Marker identification	Validation	ROC curve	Representative (provenance/ verification of state)	Reference samples	Test samples	Validation set	Confirmed by ref/ secondary method	Mixed adulterants
							Honey samples collected from different provinces of China between March and September 2014					
(Silva et al., 2017)	Triplicate analysis	N.A.	PCA: 2PCs 86,1% variance	yes	Leave one-out cross-validation	N.A.	Different producers and different sampling years.	Authentic samples obtained by certified producers	N.A.	N.A.	N.A.	N.A.
(Jandrić et al., 2015)	n = 4	N.A.	PCA: 2PCs 49,6% variance.	yes	seven-fold cross-validation; classification of an external set of samples	N.A.	Samples from different species, collected from different locations in the North and South Islands of New Zealand	Authentic honey samples obtained from honey producers	Samples with manuka, clover, khamahi and rata floral origin	33	N.A.	N.A.
(Spiteri et al., 2016)	Extraction in triplicate for Orbitrap-MS and in duplicate for TOF-MS	yes	PCA: 2PCs 36,4% variance (Orbitrap) 30,1% (TOF-MS)	yes	N.A.	N.A.	Four botanical origins, collected from different retail outlets	The samples were tested for their authenticity using pollen analysis, isotope ratio mass spectrometry and an NMR-profiling approach	N.A.	N.A.	N.A.	N.A.
(Kus & van Ruth, 2015)	Analysis in triplicate	N.A.	PCA: 2PCs 41,6% variance	yes	N.A.	N.A.	Six floral origin collected in 2010 and 2011	Floral analysis confirmed the origin	N.A.	N.A.	HPLC-DAD	N.A.
(Cubero-Leon et al., 2018)	15 from each field	yes	PCA: 2PCs 24,8% variance (ESI+) 24% (ESI-)	yes	Seven-fold cross-validation; external samples set validation; metbaolites identity confirmed with reference standards injection	yes	Carrot from different varieties, cultivated in two Belgium regions, in organic and conventional farming, over four consecutive years.	Certified organic and conventional samples were collected directly from the fields.	Carrot samples of Nerac and Namur varieties from organic and conventional farming	70	N.A.	N.A.
(Balog et al., 2016)	Each meat species divided into four pieces	N.A.	N.A.	N.A.	Leave-20%-out cross-validation in the case of minced meat patties and leave-oneanimal-out cross-validation in the case of authentic meat samples	N.A.	Five equine and five bovine samples supplied by an Irish abattoir including two Hereford Cross, two Limousin Cross, and one Blonde Cross breed. 10 Scottish bovine) samples supplied by a Scottish abattoir; all samples were from different animals	N.A:	N.A.	N.A.	N.A.	1.25%:98.75%; 2.5%:97.5%; 5%:95%; 10%:90%; 33%:67% mixtures of horse, wagyu, venison and grain beef meats
(Vaclavik et al., 2011)	Mixtures prepared in duplicate	N.A.	PCA: 2PCs 74,51% variance	yes	Leave one-out cross-validation; mixtures analyses	N.A.	Samples from different Czech producers	Pork lard and beef tallow pure samples	Mixtures prepared in the laboratory	12 admixtures samples	N.A.	Mixes of neat lard and beef tallow in the ratios 5:95, 10:90, 25:75, 50:50, 75:25, and 90:10 (w/w). Fat

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Table 3 (*continued*)

Reference	Extraction replicates	QC	Performance	Marker identification	Validation	ROC curve	Representative (provenance/ verification of state)	Reference samples	Test samples	Validation set	Confirmed by ref/ secondary method	Mixed adulterants
												isolated from pork and beef mixed in the ratios 10:90, 25:75, 50:50, 75:25, and 90:10 (w/ w)
(von Bargen et al., 2014)	N.A.	N.A.	N.A.	yes	N.A.	N.A.	Different supermarkets, date of purchase, packaging, storage conditions, end-products	N.A.	Processed samples prepared in-house and purchased from the market	N.A.	N.A.	yes
(Prandi et al., 2017)	Samples extracted in duplicate	N.A.	N.A.	yes	Analysis of blind samples	N.A.	N.A.	N.A.	N.A.	3	N.A.	80:20; 60:40; 40:60; 20:80 (% of beef:pork)
(Cajka et al., 2013)	N.A.	N.A.	N.A.	yes	Four-fold cross-validation	N.A.	Group 1: chicken fed with the feed with the addition of chicken bone meal. Group 2: chicken fed with the feed without the addition of chicken bonemeal	N.A.	Chicken fed without the "contaminated" feed six months after the model creation	16	N.A.	N.A.
(Sarah et al., 2016)	10 for each meat type	N.A.	N.A.	yes	Markers confirmation with a target LC-MS method	N.A.	Different abattoirs	N.A.	N.A.	N.A.	LC-MS target method	N.A.
(Montowska et al., 2015)	3 technical replicates	N.A.	N.A.	yes	N.A.	N.A.	Meat purchased at English and Polish supermarkets or manufactured in pilot plant	N.A.	N.A.	N.A.	N.A.	N.A.
(Oliveira et al., 2015)	2 replicates (PTR-MS)	N.A.	N.A.	N.A.	20% of the samples used for the external validation of SIMCA models	N.A.	Commercial samples of conventional, "free range" and organic productions	N.A	N.A.	8	N.A.	N.A.
(Rubert et al., 2016)	Duplicate of six samples	N.A.	PCA: 2PCs 58,7% variance (ESI+) 64,1% (ESI-)	yes	7-round internal cross-validation	N.A.	Samples collected from Spanish, Czech, Turkish markets.	10 samples labeled as PDO (Protected Designation of Origin).	N.A.	N.A.	N.A.	N.A.
(Aliakbarzadeh et al., 2016)	N.A.	N.A.	PCA: 2PCs 48,2% variance	yes	N.A.	N.A.	Samples from 8 different regions of Iran.	N.A.	N.A.	N.A.	N.A.	N.A.
(Guijarro-Díez et al., 2015)	Extraction in triplicate	yes	N.A.	yss	1/3 out cross-validation	N.A.	Samples from Spain and Iran.	Authentic samples certified according to ISO 3632 and HPLC analysis of dyes	N.A.	N.A.	N.A.	N.A.
(Black, Haughey, et al., 2016)	N.A.	yes	PCA: 2PCs 33,5% variance (ESI+)	yes	1/7 out cross-validation; analysis of commercial samples	N.A.	Samples sourced from different parts of the world	N.A.	Samples purchased at various retailers and web sites	78	yes (FTIR)	N.A.
	N.A.	N.A.	PCA: 2PCs 45,6% variance (ESI+)	yes	1/3 out cross validation	N.A.		N.A.	N.A.	N.A.	N.A.	N.A.

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Table 3 (*continued*)

Reference	Extraction replicates	QC	Performance	Marker identification	Validation	ROC curve	Representative (provenance/ verification of state)	Reference samples	Test samples	Validation set	Confirmed by ref/ secondary method	Mixed adulterants
(Vaclavik, Lacina, et al., 2011)							3 wine varieties from different geographical origins.					
(Springer et al., 2014)	N.A.	N.A.	PCA: 2PCs explained from 25% to 39% variance (ESI+)	N.A.	2 set of samples measured on different devices; 1/7 out cross validation	yes	Wine varieties from different botanical origins.	Declaration of varietal purity (100%) according to the producers.	Additional wine samples, including Riesling, Pinot Gris and Pinot Blanc origin	74	N.A.	N.A.
(Rubert et al., 2014)	Duplicate of six samples	N.A.	PCA for varieties: 2PCs 19,4% variance ESI+	yes	Seven round internal cross-validation	N.A.	Samples from 12 different countries, produced by different vineyards, from different grape varieties	Samples provided by the Federal Institute for Risk Assessment (Berlin, Germany).	N.A.	N.A.	N.A.	N.A.
(Ziółkowska, ìWąsowicz, & Jeleń, 2016)	3-5 replicates each sample	N.A.	PCA for varieties: 2PCs 67,6% variance (white wines); 92,0% (red wines)	yes	20% of samples used as evaluation set; leave-one out cross-validation	N.A.	Commercially available white wines produced in Chile, USA, France, Bulgaria, Moldova, Spain, Argentina, Australia and South Africa, and red wines in Chile, Bulgaria, California, France and Moldova	N.A.	N.A.	N.A.	GC-MS	N.A.
(Kew et al., 2017)	Three samples analyzed in replicate	N.A.	N.A.	yes	N.A.	N.A.	Samples from the 2010, 2012 and 2014 years. Scotch Whisky samples consisted of a mixture of malts (n = 55) and blends (n = 30)	Authentic reference samples provided by the Scotch Whisky Research Institute (SWRI).	N.A.	N.A.	N.A.	N.A.
(Collins et al., 2014)	Samples analyzed in triplicate	N.A.	N.A.	yes	N.A.	N.A.	37 bourbon whiskeys, 13 rye whiskeys, 6 Tennessee whiskeys and 7 other American whiskeys	N.A.	N.A.	N.A.	N.A.	N.A.
(Garcia et al., 2013)	N.A.	N.A.	Different brands: PCA: 2PCs 50,9% variance (ESI+); 23,0% (ESI-) Adulteration: PCA: 2PCs 85,7% variance (ESI+); 89,6% (ESI-)	yes	N.A.	N.A.	Scotch whiskies from different brands.	Counterfeit samples provided by the Brazilian Federal Police.	N.A.	N.A.	N.A.	25, 50, 70, 80 and 90% addition of adulterant (brazilian distilled whisky)
(Hurkova et al., 2017)	N.A.	N.A.	N.A.	yes	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
(Hrbek et al., 2017)	N.A.	yes	PCA: 2PCs 52,8% variance (ESI+) 68,3% (ESI-)	yes	*k*-fold internal cross-validation, analysis of certified reference materials of non GMO samples	N.A.	different varieties grown in different areas and agricoltural conditions.	Samples provided by the Crop Research Institute (CRI) of Prague.	N.A.	N.A.	N.A.	1%,5% and 10% of GMO addition to the CRM material

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Table 3 (*continued*)

Reference	Extraction replicates	QC	Performance	Marker identification	Validation	ROC curve	Representative (provenance/ verification of state)	Reference samples	Test samples	Validation set	Confirmed by ref/ secondary method	Mixed adulterants
(Pastor et al., 2016)	Three samples from every cereal flour sample	N.A.	N.A.	yes	N.A.	N.A.	23 small grain samples and 17 corn hybrid samples	Samples obtained from the Institute of Field and Vegetable Crops BNS Seme,ˆ Novi Sad, Serbia	N.A.	N.A.	N.A.	N.A.
(Prandi et al., 2012)	Calibration curve prepared in triplicate	N.A.	N.A.	yes	Analysis of home made mixtures and commercial samples	N.A.	Wheat varieties were provided by Società Produttori Sementi SpA	N.A.	Mixtures and commercial samples analyses	N.A.	N.A.	5%, 20%, 56%,70%, 75%, 90% of common wheat addition
(Geng et al., 2016)	N.A.	N.A.	PCA: 2PCs 90,2% variance	yes	1/7 out cross-validation	N.A.	For every sample flour, bread crumb and bread crust were analyzed	N.A.	N.A.	N.A.	N.A.	N.A.
(Righetti et al., 2016)	Double extraction of 8 samples (10% of the samples set)	yes	PCA: 2PCs 50% ESI+ and 47.2% ESI- explained variance	yes	Leave one-third out cross- validation	N.A.	Three varieties, two growing locations, two farming conditions, two harvest years.	N.A.	N.A.	N.A.	N.A.	N.A.
(Matthews et al., 2012)	Triplicate extractions	N.A.	N.A.	yes	Seven fold cross-validation	N.A.	45 varieties from common and durum wheat	Wheat seed provided from Colorado State University	N.A.	N.A.	N.A.	N.A.
(Righetti et al., 2018)	N.A.	yes	PCA: 2PCs > 52% variance for both ESI+ and ESI- models	yes	Markers confirmed with a new metabolomic experiment. Analysis of mixtures in duplicate	yes	2 locations, 2 agricultural conditions, 2 harvest years	Samples from Odisseo (durum, n = 26) and Blasco (common, n = 26) wheat lines	Samples from five varieties of durum (Triticum durum Desf.) and common wheat (*Triticum aestivum* L.), grown with conventional and organic farming	173	N.A.	1%, 2%, 3%, 5%, 10%, 15% addition of common wheat to durum wheat
(Rubert et al., 2017)	N.A.	yes	PCA: 2PCs > 58% variance for both ESI+ and ESI- models	yes	1/7 out cross-validation	N.A.	Valencian Tiger nuts and African tiger nuts from Niger, Burkina Faso and Mali	All the Valencian tiger nuts were PDO samples	N.A.	N.A.	N.A.	N.A.

N.A.: information not available in the paper.

the effect of nuisance variation (Bevilacqua et al., 2017). However, quite often in non-targeted methods the number of experimental variables usually greatly exceeds the number of objects, especially with the development of new mass spectrometry-based technologies. Variables, i.e. metabolites or proteins, are represented by mass/retention time combinations and it is typical to have a huge number of features varying from several hundred to many thousands, depending on the experimental and analytical conditions. This increase in experimental possibilities, however, does not correspond to a proportional increase in the number of samples, leading to a serious complication for the statistical analysis, including the risk of type I and type II errors and thus lowering the prediction power (Franceschi, Vrhovsek, Mattivi, & Wehrens, 2012). Power analyses, that are an important aspect of experimental design, are often avoided and sample size determination seems to be driven by sample availability, laboratory practice or extrapolated from the existing literature. Approaches developed in other fields (Blaise, 2013) allow an efficient *a priori* evaluation of the number of samples to be included in a study in order to identify statistically significant variations throughout the data set. Indeed, power laws, analysis of variance (ANOVA) and ANOVA Simultaneous Component Analysis (ASCA) (Khakimov, Gürdeniz, & Engelsen, 2015) (Smilde et al., 2005) can be used to decide on the minimum number of samples required (Blaise et al., 2016). On-line software tools are also available for simple power/sample size calculations such as G*Power (http://www.gpower.hhu.de/en.html) or Glimmpse (https://glimmpse.samplesizeshop.org/#/) (Kreidler et al., 2013). Despite recent efforts (Blaise, 2013) (Blaise et al., 2016), there is still no widely accepted criteria for sample size determination even though it is necessary to gain confidence in the results generated.

Apart from the size of the data set, samples have to be representative and biological variation should be accurately defined. An appropriate number of biological replicates have to be included in each group to confidently answer the question in a statistically robust manner. Samples should be selected minimizing the non-controlled variations among them as much as possible (such as storage conditions) checking also that groups are balanced (Xia, Broadhurst, Wilson, & Wishart, 2013) (that is, when the number of samples in different classes do not vary greatly). Ideally, we would measure one source of variation while controlling all other sources of variation. For example, when identify the metabolic changes resulting from the agricultural regimen of tomatoes, all source of variation must be controlled, such as genotypes, environmental factor, years of harvesting. By controlling other sources of variation, we are confident that the observations are due to the parameter we are testing, that means the organic/conventional condition. However, when performing an open field study, it is difficult to control these sources of variation and so instead we need to ensure the variability is equivalent between different groups of tomatoes. For example, we have to ensure the range of tomatoes varieties, growing locations and harvesting years is equivalent in the samples group because we know that this will influence the measured metabolome. So, when investigating the differences between a group of tomatoes cultivated in organic and in conventional conditions, and the organic samples are from Spain while the conventional are cultivated in Netherlands, there are two sources of variations, agricultural conditions and geographical origin. Where these sources of variation are not matched then the metabolic changes observed are a result of a combination of the two or more of them. If the geographical origin of tomatoes is similar within the groups, then the influence is removed and the study now has only one sources of variation, the conventional/organic regimen.

In any authenticity research, “authentic” samples are essential in order to understand the natural variation within a population. These samples can be provided by producers or prepared by researchers under controlled conditions. Also, the adulterated samples could be formulated ‘fit for purpose’, e.g. by dilution (relevant in wine or juice authentication) (Rubert, Zachariasova, & Hajslova, 2015).

One of the critical steps in non-targeted approach is the sample preparation which has hugely important consequences on the accuracy of the analytical results produced. Ideally, no sample preparation would be required for the analysis of samples as every manipulation might influence the reproducibility. This is feasible only for some matrices (i.e. wine) or by using direct analysis techniques, such as DART (Rubert, Lacina, Fauhl-Hassek, & Hajslova, 2014), Rapid Evaporative Ionization Mass Spectrometry (REIMS) (Balog; et al., 2016) and PTR-MS (Granato, Koot, & van Ruth, 2015) (Black, Chevallier, & Elliott, 2016).

In order to further increase the number of detected molecules (thousands), both positive and negative ionization modes should be performed. Therefore, sample handling should be minimal, non-selective, aiming to get coverage of a wider range of analytes by simple preparation steps. As no single analytical technique is suitable for the detection and identification of the “true fingerprints”, multiplatform approaches represent the best solution. In this context, sample pretreatment should be the minimum possible to make it compatible with the instrumental techniques.

1.3. Data processing to extract meaningful markers

For processing massive information based on separation techniques and mass spectrometry, effective software tools capable of rapid data mining procedures must be employed. Note that data matrices contain thousands of variables (*m/z*, retention time, intensity), and they have to be converted into more manageable information (Bevilacqua et al., 2017). According to the literature review, metabolomic approaches are the most often applied. In these type of studies, data processing and data pretreatment must be carried out in order to permit the identification of significant compounds, which capture the bulk of variation between different datasets and may therefore potentially serve as biomarkers (Riedl et al., 2015). Data processing usually involves four basic steps: deconvolution, alignment, filtering and gap filling (Riccadonna & Franceschi, 2018) (Mastrangelo, Ferrarini, Rey-Stolle, García, & Barbas, 2015). The features, defined by their *m/z* and retention time, and their intensities in different samples are used for the statistical analysis. Samples can be grouped and can be observed using score plots, heatmaps or hierarchical clustering. After data pretreatment, a statistical comparison can be performed using the multivariate data analysis (MVDA). Usually this step involves unsupervised models (PCA) and supervised classification tools, such as PLS-DA and OPLS-DA (Franceschi et al., 2012). These supervised methods are performed to maximize differences between groups and to highlight potential biomarkers.

1.4. Validation

In food authenticity studies, non-targeted approaches results end up with a prediction of class belonging or with the validation of discriminative models and thus identification of few markers. To verify the relevance of modelling results, appropriate model validation is essential for non-targeted approaches, but it is often found to be used insufficiently or inconsistently.

As example, only 35% of the articles presented in this opinion mentioned the use of an external set of samples for the validation of the model while 25% of papers do not perform any validation study (not even a cross-validation study). Furthermore, when target compounds are identified, only 12% of the articles clearly indicated that the relative reference standard were injected. In addition, only 10% of works performed an ROC curves evaluation and only 30% of papers monitored the analytical variability by using quality control (QC) samples.

1.4.1. Analytical validation

The first type of validation that should be considered is the analytical one, which usually include the randomization of the injection samples order and the quality control samples (QCs). QCs samples are a

pool of equal volumes of each sample of the set. These mixtures must be injected at the beginning of the run to equilibrate and stabilize the system as well as at regular intervals throughout the sequence run (i.e. every 10 injections). For non-targeted analysis, the quality of the instrumental performance is checked by the tight clustering in the center of the plot of QC in the preliminary PCA (Godzien, Alonso-Herranz, Barbas, & Armitage, 2015). The US FDA has proposed other useful criteria for analytical method validation (Food and Drug Administration, 2001) to calculate the relative standard deviation (RSD) of each analyte in the QCs: features with RSD% higher than 40% should be filtered out, since they would not be good candidates as markers.

So far, only a few studies presented in literature mentioned the preparation and evaluation of QCs, either showing the related scores plots, that is a fundamental evidence of a good analytical procedure. In addition, the use of an internal standard (Want et al., 2013) is strongly recommended, even though not largely applied in the food fraud field as yet. By adding this compound(s), the performance of the extraction procedure and the chromatographic run can be checked, and it could also be used as an additional tool for post processing data evaluation (i.e. normalizing the peaks area with the Internal Standard ones) (Khamis, Adamko, & El-Aneed, 2017). In the research presented by Khamis et al., for example, a deuterated internal standards is used to compensate the matrix effect and the relative ion suppression. Moreover, in the work of Ulaszewska et al., $^{13}C_1$ labeled creatinine and trans cinnamic acid-d_5 were used in urine metabolomics as additional control of the extraction efficiency (Ulaszewska et al., 2016). Recently, Boysen et al. presented a workflow, called best-matched internal standard (B-MIS) normalization, that should lead to normalize peaks signal according to the isotope-labeled internal standards that have a similar behavior during the analysis (Boysen, Heal, Carlson, & Ingalls, 2018).

1.4.2. Internal validation

Multivariate approaches suffer from overfitting, thus validation is an obligatory component of any analysis. Typically, cross-validation approaches are used, in which a proportion of the data (e.g., 10–40%, the “validation set”) are randomly removed, and the model is built with the remaining “training set”. This procedure is repeated many times until each sample has been in the test set exactly once (leave-*n*-out procedure). The accuracy of the model on these left-out samples gives an estimate of the predictive power for unseen samples and also the robustness of the model to perturbations of the data.

Model performance is usually described by the goodness-of-fit parameter (R^2X), the proportion of the variance of the response variable that is explained by the model (R^2Y) and the predictive ability parameter (Q^2). Most of the papers investigated through this review reported quite nice prediction abilities. Indeed, Rubert et al. while investigating the wine authentication presented values always higher than 0.7 in all the supervised models created (Rubert et al., 2014). Another interesting example is the Oregano study performed by Black et al. in which the three parameters are always higher than 0.9 in all the models created (Black, Haughey, Chevalier, Galvin-King, & Elliott, 2016) indicating excellent classification performance as well as prediction ability.

In a few studies, also permutation testing and Monte Carlo simulation were used as a tool to avoid overfitting (Riedl et al., 2015). In addition, sensitivity (percentage of samples correctly classified) and specificity (percentage of samples correctly rejected) are used to evaluate the classification performance (USP Pharmacopeial Convention, 2016, pp. 2053–2067). The visual tool is represented by the receiver operator characteristic (ROC) curves, that has not been extensively applied in food studies (Righetti et al., 2018; Springer et al., 2014), but widely elsewhere (Xia et al., 2013). Recently (Righetti et al., 2018), this tool was employed to verify the reliability of markers to identify the durum what aduteration in a confirmatory study. The authors reported area under the curve (AUC) for the most significant markers values ranging from 65% to 100% (Righetti et al., 2018), and thus being classified as excellent markers.

1.4.3. External validation

Evaluating the repeatability and the performance of a model is an invaluable and crucial step before the introduction of this new model in routine practice. Independent external validation should be performed by the assessment of an external set of samples that were not used for model building. Samples should be critically selected in order to demonstrate the validity of the model and expand the application of the method, modelling all the possible sources of variability of the considered matrix. Therefore, for example different geographical origins, growing seasons, cultivars, and producers have to be considered and included in the building of the model.

Interesting examples of this approach can be found in the articles studied for this opinion.

In the honey floral origin discrimination performed by Jandric' et al., 33 samples from 4 different botanical origin were used for the model validation (Jandrić et al., 2015). Moreover, the geographic origin discrimination between different Spanish Extra Virgin Olive Oils (EVOO) presented by Gil-Solsona et al. was validated with 15 samples from cultivars representative of all the Spanish EVOOs and collected in a different season with respect to the samples used for the model creation.

Finally, a complete confirmatory metabolomic study with an higher amount of samples and with the introduction of more sources of variability was executed by Righetti et al. in order to confirm the markers selected in the preliminary study. (Righetti et al., 2018).

1.4.4. Marker validation

If the ultimate goal of the non-targeted approach is to move markers from the research laboratory to the food authorities control routine practice, the significance of the markers must be confirmed:

- During the external validation study, where more sources of variability are considered;
- Evaluating the marker performance with the area under the curve (AUC) of the ROC;
- By a survey of blind real samples;
- By the analysis of admixture samples (that are samples in which authentic sample is mixed up with different percentage of adulterant), especially if a legislation level is established for the target fraud.

Another step of validation can be considered the integration of multiplatform data as well as data fusion. Indeed, multiplatform characterization of food samples with subsequent data fusion has been shown to improve prediction ability of multivariate models in authenticity testing (Biancolillo, Bucci, Magrì, Magrì, & Marini, 2014). The concatenation of analytical information from complementary instrumental techniques can be established on different levels. Data fusion is becoming more and more important in food authentication but appropriate preprocessing and model validation are required (Riedl et al., 2015) (Biancolillo et al., 2014).

In this context, ring trails are highly recommended to assess the reliability of non-targeted approaches across different laboratories. To the best of our knowledge, they have not been applied yet in the food fraud analysis, but some attempts of “metabo-ring tests” were reported recently in literature (Martin et al., 2015) (Cajka, Smilowitz, & Fiehn, 2017). Bringing together different mass spectrometers across Europe, the authors obtained consistent results and interestingly, no effect of the LC-MS instrumentation (TOF, QTOF,LTQ-Orbitrap) was reported. It should be noted that the Standard Operating Procedure (SOP) for sample preparation and specific statistical design were the same and undoubtedly played an important role in the quality of the study. In the work presented by Martin et al., eleven different mass spectrometers

were used for two tests: in the first one two groups of urine samples were analyzed, one of them spiked with 32 standard metabolites. Interestingly, all the mass spectrometer instruments were able to discriminate between the two groups of samples and most of the spiked compounds were identified as features responsible of the clusterization. In the second test, blood samples collected from rats fed with low or high Vitamin D diets were analyzed. The separation of the two groups was not satisfactory but it was due to the low biological contrast of the two groups and not to differences through the platform. Moreover, the trends detected with different instruments were comparable (Martin et al., 2015).

In the work presented by *Cajka* et al., nine mass spectrometers were used to perform a non targeted lipidomic study on human plasma samples. The classification results obtained were in good agreement and the most discriminative lipids found by each instrument overlapped in the 92% of the cases.

Coming back to the food field, however, large amounts of samples are very difficult to find and specific ring tests for each target fraud (and for each commodity) should be developed.

Another opportunity is to make the collection of massive amounts of non-targeted data available to all investigators who are interested in undertaking analysis. As an example, "MetaboLights" is an on-line repository where, for each study, data are shared by following a data-protocol deposition procedure to fully detail the experiment (Kale et al., 2016). The database is cross-species and cross-technique and covers metabolite structures and their reference spectra, as well as their biological roles, locations, concentrations, and experimental data from different experiments. If data for the food fraud of interest can be shared, the raw files could be potentially used as independent data set for an external validation: the model should be able to correctly cluster these samples, even if probably the number of extracted features will not be exactly the same. Additionally, if the non-targeted study aims to identify relevant markers, the shared results could be helpful to identify robust compounds potentially already detected in different laboratories or, if not, to merge complementary results. A possible step in this direction would be to make data-protocol-algorithm deposition a prerequisite for publication in all peer-reviewed scientific journals.

1.5. Applicability in official and legal trials

Official procedures require a high degree of result reproducibility across different laboratories, instrumentation and analysts. So far non-targeted method have been reported to be mainly "in-house" developed and validated, with little focus on inter-laboratory reproducibility. For this reason, standardization of analyses performed at different laboratories will be a challenge, but it should be recognized as essential to allow data to be more widely comparable. To take advantage of some recent analytical breakthroughs, the combination of non-targeted and semi-targeted strategies is strongly recommended to provide a robust approach for a short but well defined list of compounds. Due to their differing objectives, the application of non-targeted methods for quantitative or confirmatory purpose remains challenging. Yet these approaches should be considered essential to select and identify significant markers, mainly when the adulterant is unknown. However, once markers are identified, optimized methods should be applied for the accurate quantification required by a confirmatory purpose, as recently presented by different research groups (Wielogorska et al., 2018) (Jandric, Islam, Singh, & Cannavan, 2017). Subsequently, these 'biomarker target methods' can be shared and applied through different laboratories.

To summarize, in order to be able to present untargeted analysis in legal trials and have them accepted, non-targeted models must be fully validated as detailed in this article ensuring that identified compounds are highly specific of the food fraud detected.

If the non-targeted approach ends with a prediction cluster, only unknown samples having an adulteration level in line with those used to build the model (e.g. that are classified in a specific "not-compliant cluster") can be declared as fraudulent and thus illegal.

2. Conclusions

This Opinion has set out to summarize the scientific activities published up to now on the non-targeted mass spectrometry approaches to food fraud detection. The authors have outlined a possible approach for the development and validation of these types of methods, taking into account that at the moment there is no harmonized, agreed or 'official' workflows. Additionally, global considerations on the applicability of these methods for legal purposes are provided.

Processes harmonization does appear to only be at the beginning and both public and private institutions will have to increase their efforts in order to finalize a shared approach, able to guide the development of robust non-targeted methods for food fraud detection using spectrometric techniques.

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